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14. ABSTRACT:

Malaria is the primary infectious disease threat facing the U.S. solider, and is the leading cause of all casualties during tropical deployments. The long-term objective of this project is to identify and prepare the malaria parasite forms causing severe anemia, and then apply functional genomics and bioinformatics tools to identify 15 to 30 proteins that could form the basis for an effective vaccine at both the pre-erythrocytic and blood stages of malaria infection. The project will then evaluate these lead candidates for their recognition by sera collected from immune individuals, in order to identify the leading 3 to 5 candidates for a blood stage vaccine that prevents severe malarial anemia.

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INTRODUCTION: Malaria is the primary infectious disease threat facing the U.S. solider, and is the leading cause of all casualties during tropical deployments. The long-term objective of this project is to identify and prepare the malaria parasite forms causing severe anemia, and then apply functional genomics and bioinformatics tools to identify 15 to 30 proteins that could form the basis for an effective vaccine at both the pre-erythrocytic and blood stages of malaria infection. The project will then evaluate these lead candidates for their recognition by sera collected from immune individuals, in order to identify the leading 3 to 5 candidates for a blood stage vaccine that prevents severe malarial anemia.

BODY: We have made good progress in many activities during our second year as described below.

Human Subjects Research Protocol

Our DOD protocol titled, "Antigens for a Vaccine that Prevents Severe Malaria" describes the functional genomics and immunoreactivity studies that we are performing on malaria parasites. After extensive revisions during the first grant year, it now supports laboratory work being conducted only at the Seattle Biomedical Research Institute in the U.S., and includes sample processing, immunoparasitology, and functional genomics studies on samples collected under a separately IRB-approved longitudinal cohort study. The longitudinal cohort study is supported by funds from the National Institutes of Health and the Bill & Melinda Gates Foundation.

We submitted the DOD protocol for continuing review to our local IRB (Western IRB in Olympia, WA) in December 2006 and received continuing approval on January 2, 2007.

Optimization of Laboratory Methodology

Microarray Printing We have improved the quality of our oligonucleotide spotted microarrays by optimizing humidity and pin strike speeds as well as adjusting oligonucleotide concentrations. We have implemented quality control analyses to ensure quality and consistency of slides between different prints using open source (Bioconductor) and commercial (Acuity) programs.

RNA Extraction and Processing RNA extraction and purification from whole blood samples collected in Tanzania was improved and optimized. The RNA extraction time was reduced from 12 hours to 90 minutes and a procedure was added to remove globin mRNAs produced by reticulocytes, which compromises microarray sensitivity.

DNA Microarray Studies of Severe Anemia Parasites

Our microarray studies continue to focus on comparing *P. falciparum* gene expression in children with severe malarial anemia and infected children without anemia. So far we have selected samples from 10 anemic and 13 non-anemic patients for our study. The RNA processed from these patients' blood samples were co-hybridized with reference RNA on a total of 73 microarray slides. The reference RNA pool was created from 22 different *P. falciparum* infected patient samples and two asynchronous lab strains, which enables us to compare gene expression between two different groups of slides.

PfEMP-1 proteins, encoded by *var* genes, are associated with disease states and exported from the parasite to the infected erythrocyte cell surface. Our microarray studies have thus far identified six *var* genes that are transcribed at significantly higher levels by parasites in anemic

versus non-anemic patients. This study confirms the upregulation of *PFL0030c* seen in pilot microarray studies and initial qPCR experiments.

Non-*var* genes with PEXEL, VTS, and transmembrane motifs would also make good vaccine antigen candidates. We have identified nine such genes as being upregulated in parasites from anemic vs. non-anemic patients.

qPCR experiments are being conducted to confirm microarray results.

Proteomics Studies of Severe Anemia Parasites

We have launched MS/MS studies to compare the abundance of surface proteins on parasites collected from children with different clinical syndromes. Nine samples have been analyzed by LC-MS/MS, including 2 samples from children with hyperparasitemia complicated by moderate anemia and one case of severe malarial anemia without hyperparasitemia, as well as 6 cases of uncomplicated malaria used for comparative purposes. The same samples are currently undergoing analysis by quantitative proteomics using FTICR-MS. The results of these studies will identify the subset of surface proteins that are expressed at higher levels in severe anemia parasites than other parasites. If these pilot studies are successful, we will expand this approach to a larger number of samples.

Separately, we have expressed the conserved cytoplasmic region of PfEMP1 and we are in the process of eliciting antibodies to this protein fragment. Specific antisera to the conserved region of PfEMP1 will be used for direct analysis of PfEMP1 forms expressed by parasites causing malarial anemia, both by Western blot and by immunoprecipitation.

Future Directions

Currently we have six additional parasite samples from infected children with associated clinical information that will be used to perform array analysis in the next two months. Our birth cohort included 1,045 children born in the Muheza area in Tanzania, from 2002 to 2005. Blood was drawn on a regular basis as well as during malarial episodes after birth, and these samples form the repository of parasites and sera from which we are able to conduct our IRB-approved studies funded by DoD. Clinical information such as parasitemia count and hemoglobin level was determined for each blood sample. Additional samples from our repository will be processed and used for microarrays as our analysis of the cohort blood smear and hemoglobin data identifies additional suitable material for this study.

Var genes are highly variable between strains and so for future experiments, we may expand the repertoire of microarray *var* gene probes as more is learned about *var* genes, especially as new isolates of *P. falciparum* are sequenced.

We have started to express recombinant forms of the proteins that are preferentially expressed by severe anemia parasites. We have established expression platforms including *E. coli* expression and cell-free expression using the ENDEXT technology to express the recombinant molecules. These proteins will be used for serosurveys to identify whether antibodies against these proteins correlate with protection from severe anemia. The serosurveys will use sera from our repository that include samples already obtained from longitudinal cohort studies of Tanzanian children in Muheza.

KEY RESEARCH ACCOMPLISHMENTS: Below is a list of key research accomplishments emanating from this research:

- Human Subjects Protocol Continuing Review Approvals obtained
- Identified several known surface protein genes (called *var* genes) that are upregulated in parasites causing anemia
- Identified several hypothetical protein genes that are upregulated in parasites from anemic patients that would also make good vaccine antigen candidates
- Established cell-free protein expression in our laboratory using ENDEXT technology to prepare recombinant proteins for serosurveys
- Improved the quality of our oligonucleotide spotted microarrays and implemented quality control analyses to ensure quality and consistency of slides between different prints
- Continued to improve RNA stabilization and extraction assays

REPORTABLE OUTCOMES:

Publications

1. **Duffy PE**, Fried M. 2006. Red blood cells that do and red blood cells that don't: how to resist a persistent parasite. **Trends Parasitol**, 22(3):99-101
2. Kappe SHI, **Duffy PE**. 2006. Malaria liver stage culture: *in vitro veritas?* **Am J Trop Med Hyg**, 74(5):706-7
3. **Duffy PE**, Mutabingwa TK. 2006. Artemisinin combination therapies. **Lancet**, 367(9528):2037-9.
4. Ntoumi F, Kwiatkowski DP, Diakite M, Mutabingwa TK, **Duffy PE**. 2006. New interventions for malaria: mining the human and parasite genomes. **Am J Trop Med Hyg**, in revision.
5. AV Oleinikov, E Rossnagle, S Francis, TK Mutabingwa, M Fried, **PE Duffy**. 2006. Effects of gender, parity and antigenic variation on seroreactivity to candidate pregnancy malaria vaccines. **J Infect Dis**, accepted.

Presentations

1. Invited Speaker, "Malaria vaccines", Science & Technology Roundtable. Seattle, Washington. 10 February 2006
2. Seminar Speaker, "Tropical Diseases Research", Seattle Pacific University, Natural Sciences Seminar. 13 February 2006.
3. Symposium Speaker. "Malaria Pathogenesis and the Parasite Genome." Keystone Symposia, Malaria: Functional Genomics to Biology to Medicine. Taos, New Mexico. 28 February – 5 March 2006
4. Seminar Speaker. "Malaria at the mother-child interface: epidemiology, pathogenesis, interventions." NIH Seminar Series. Bethesda, Maryland. 28 March 2006.
7. Invited Speaker. President's Malaria Initiative: Research into Practice Meeting. Kampala, Uganda. 25-28 April 2006.
8. Medicine Grand Rounds Speaker. "Malaria Pathogenesis and a Vaccine." University of Tennessee, Memphis. 21 June 2006.
9. Keynote Speaker. "Pregnancy Malaria: Two Parasites and an Inflamed Host." ICOPA XI. Glasgow, Scotland, United Kingdom. 6-11 August, 2006.

CONCLUSION: We have identified a number of known surface proteins as well as hypothetical proteins that are associated with malarial anemia parasites, using microarray and proteomics tools. These functional genomics studies have focused on parasite samples that had been processed and stored immediately after collection from Tanzanian donors, and maintained in our repository. These encouraging initial results support our primary hypothesis for the

proposed studies, and will continue to be extended with a larger number of samples. We are now beginning the process of assessing these proteins as targets of protective immune responses as well as candidates for severe malaria vaccines as described in our original DOD proposal. We have started to express recombinant forms of these proteins, and in the coming year will launch seroepidemiology studies that examine whether antibodies against these proteins correlate with protection from severe anemia.

REFERENCES: None.

APPENDICES: Publications 1, 2, 3, and 5.

SUPPORTING DATA: None.

opportunities. In addition, specific parasitology projects are being undertaken. For example: (i) the network has supported the analysis and dissemination of the results of the scabies-mite expressed sequence tag collection; (ii) network-supported researchers are developing databases that focus on the biology and ecology of parasites to complement available genome databases; and (iii) the network has recently made available a microarray service that includes expert assistance and access to microarray databases and analytical tools (http://vbc.med.monash.edu.au/~powell/vbc-microarray/vbc-microarray-capabilities.html). These initiatives are the result of a close collaboration between the ARC/NHMRC Network and the Victorian Bioinformatics Consortium (VBC) – the network provides funding for personnel and the VBC provides the required infrastructure and expertise in bioinformatics.

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Red blood cells that do and red blood cells that don't: how to resist a persistent parasite

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Sixty years ago, Haldane proposed that certain abnormalities in red blood cells could be selected as malaria-resistance genes. Population studies have confirmed that many human polymorphisms confer resistance to severe malaria, although the mechanisms of protection remain unknown. A recent article proposes a new mechanism for explaining the protective effects of hemoglobin C (HbC). HbC-containing red blood cells have modified displays of malaria surface proteins that reduce parasite adhesiveness and could reduce the risk of severe disease.

The malaria hypothesis

The epidemiology of malaria can give important insights into the host and parasite factors that contribute to the pathogenesis of this disease. For example, the innate resistance of Africans (who lack the Duffy antigen on the red blood cell surface) to vivax malaria inspired the hypothesis that *Plasmodium vivax* requires the Duffy antigen for erythrocyte invasion – a notion that has been proved in subsequent studies [1].

More famously, the coinciding geographic distributions of malaria transmission and the thalassemias prompted Haldane to propose the 'malaria hypothesis', which stated that common abnormalities in red blood cells have been selected because of the fitness advantage they confer against malaria [2]. Haldane's speculation centered on the frequency of thalassemia in Mediterranean populations, for which he predicted that the deleterious effects of the homozygous state would be balanced by the increased fitness afforded to heterozygous individuals by resistance to malaria – hence manifesting a 'balanced polymorphism'. This increased fitness is commonly presumed to mean protection from the deadly malaria syndromes (e.g. cerebral malaria and severe malarial anemia), which are thought to kill at least one million African children each year [3].

'Sickle hemoglobin' (HbS) is the best-known abnormality of red blood cells that is associated with protection from malaria. HbS decreases the risk of severe malaria by >90% in some populations [4], but in its homozygous form it is fatal early in life without modern treatment. Other hemoglobinopathies {e.g. hemoglobin C (HbC) [5,6] and hemoglobin E (HbE) [7]} and deficiencies in red blood cell enzymes (e.g. glucose-6-phosphate dehydrogenase deficiency [8]) have also been linked to protection against severe malaria. The population-based studies relating abnormalities in red blood cells to protection have supported Haldane's initial hypothesis.

Mechanisms of resistance to malaria

It is unclear how any of these abnormalities in red blood cells can confer protection. Haldane speculated that the smaller erythrocytes of thalassemics, which are resistant to osmotic lysis, might also be 'more resistant to attacks by the sporozoa which causes malaria' [2]. Other researchers have obtained data supporting several mechanisms by

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which red blood cell abnormalities might confer or be associated with resistance, such as reduced parasite invasion of red blood cells, diminished intraerythrocytic growth of parasites [9], enhanced phagocytosis of parasite-infected erythrocytes (IEs) [10,11] and enhanced immune responses against IEs [12]. In the case of enhanced immunity, thalassemia increases the incidence of clinical vivax and falciparum malaria during early life [13], raising speculation that this early experience could function as a natural vaccine that affords immunity to severe malaria in later life. Although there is evidence supporting several proposed mechanisms by which various resistance genes could confer protection against malaria, none of it is conclusive.

PfEMP1 and HbC

Recently, a collaborative team of North American and African scientists led by Thomas Wellems has enlivened these interesting but, as yet, inconclusive discussions with a new proposal [6]. Earlier, this group had found that HbC is associated with resistance to severe, but not uncomplicated, malaria among the Dogon people of Mali [5]. In their recent work, they observed that HbC modifies the quantity and distribution of the variant antigen *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) on the IE surface. PfEMP1 has been implicated in numerous IE adhesive interactions and, in their detailed studies, the authors showed that HbC reduces the level of IE adhesion to endothelial monolayers, in addition to IE rosetting (the adhesion of IEs to uninfected erythrocytes) and IE agglutination by sera. They conclude that HbC might protect against severe malaria by mitigating the obstruction and inflammation caused by the PfEMP1-mediated adherence of IEs.

Barring the possibility that PfEMP1 display on HbC cells is artifactually modified during *in vitro* culture, the results obtained by Fairhurst *et al.* [6] suggest three potential scenarios: modified PfEMP1 display on HbC cells (i) reduces the risk of disease by decreasing sequestration; (ii) reduces the risk of disease without decreasing overall sequestration; or (iii) does not reduce the risk of disease *per se* but is one manifestation of the HbC phenotype that mediates protection through other mechanisms.

Resistance genes and malaria epidemiology

Which scenario best fits the HbC data? Logically, reduced parasite adhesiveness would reduce parasite sequestration and, thereby, reduce the risk of disease in the first scenario because the mass of sequestered parasites is thought to be related to severe-malaria syndromes. However, sequestration is also believed to enhance parasite survival by enabling IEs to avoid clearance from the spleen, and any effect of HbC to reduce sequestration would presumably reduce parasite fitness. Because numerous epidemiology studies [5,14–16] have failed to identify a major impact of HbC on the frequency or density of parasitemia in naturally exposed populations, the first scenario is problematic.

Fairhurst *et al.* [6] suggest that the effects of HbC on PfEMP1 display could mitigate obstruction and inflammation from the adherence of parasitized erythrocytes in the microvasculature. This would leave open the possibility that the degree of sequestration is unchanged but that the ensuing obstruction and inflammation are altered in ways that reduce the risk of disease; this would be consistent with the second scenario. It has been proposed that PfEMP1 has multiple roles in malaria pathogenesis, including cytoadhesion, adsorption of Ig to the IE surface, sequestration, antigenic variation and immunoevasion, and immunostimulation. The modified display of PfEMP1 might impact these various properties to different degrees, with the effect, for example, of mitigating inflammation without reducing sequestration, but this concept remains to be confirmed.

Interestingly, the degree to which homozygous HbC modified PfEMP1 display on the IE surface varied between parasite isolates [6]. The authors noted that protection against malaria by HbC might, therefore, be strain dependent. If HbC specifically impairs PfEMP1 expression by parasite strains (or forms) that cause severe malaria, it would be consistent with observed malaria epidemiology; although the parasite forms that cause severe malaria could experience impaired sequestration and reduced growth in HbC cells, other parasite forms might grow unimpeded, thereby accounting for reductions in severe malaria but not in parasitemia. This explanation would also fit the second scenario, in which modified PfEMP1 display on HbC cells reduces the risk of disease without reducing overall sequestration (instead, reducing only the sequestration of virulent parasites).

The major shortcoming of this explanation is the data indicating that every IE adhesive interaction measured by Fairhurst *et al.* [6] was substantially and significantly impaired for different parasite isolates that infect HbC-containing red blood cells. These data support the notion that PfEMP1 mediates these *in vitro* adhesive phenomena, in that the degree to which adhesion was impaired corresponds to the degree to which PfEMP1 display was modified. However, the substantial defect in the adhesion of HbC IEs [6] does not manifest as a reduction in parasitemia in naturally infected persons [5,14–16], indicating either that adhesion is not essential for parasite survival (an unappealing notion because mature IEs have not been reported to circulate in the peripheral blood of individuals expressing HbC) or that the *in vitro* measurements of adhesion are a poor proxy of *in vivo* sequestration.

In addition, an important issue of malaria epidemiology in HbC individuals remains to be resolved. In their clinical studies [5], Wellems and colleagues found that HbC is associated with protection from severe malaria but not from uncomplicated malaria. In a larger case-control study in Burkina Faso, Modiano *et al.* [17] observed that HbC is related to protection from both severe malaria and uncomplicated malaria. The confirmation of either epidemiological pattern would be valuable for understanding the molecular basis of HbC protection, although it is possible that HbC is associated with distinct clinical benefits in different populations [4]. This possibility raises questions about the role of HbC in the causal pathway of protection, or epistasis between HbC and other genes to control the resistance phenotype.

Answers and questions

Wellems and colleagues have opened a new avenue of inquiry for understanding red blood cell abnormalities and their effects on malaria resistance. These studies show that HbC modifies PfEMP1 display and impairs IE adhesion *in vitro*. However, their report [6] also raises several questions. For example, how well does *in vitro* adhesion correspond to *in vivo* sequestration? What is the role of sequestration in parasite survival and disease? Most specifically, how can a red blood cell abnormality impair *P. falciparum* growth or adhesion without modifying the frequency and density of parasitemia?

Whether HbC alters the host in other ways remains unknown. Malaria parasitemia frequently occurs without symptoms, and morbidity and mortality due to malaria are associated with specific host response patterns such as inflammatory cytokine responses. Although red blood cell abnormalities imply an impact on parasite biology and fitness, they might also modify the host response to infection in ways that reduce the risk of disease without reducing parasitemia. Altered display of PfEMP1 could modify the host response, as suggested by Fairhurst *et al.* [6], or individuals with HbC might have intrinsic differences in their response to infection; it is also possible that both effects occur. Nearly 60 years after Haldane proposed his inspired hypothesis, the molecular bases by which resistance genes confer protection remain a subject of intense interest and a potential pathway to new therapies against severe malaria.

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Letter

Further thoughts on where we stand on the autoimmunity hypothesis of Chagas disease

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The notion that Chagas disease could be autoimmune in nature has stirred controversy since it was proposed in the mid-1970s. In his recent *Trends in Parasitology* article [1], Kierszenbaum eloquently describes and critiques key experimental results supporting and refuting an autoimmune mechanism of pathogenesis, as he has done in several other reviews of a similar nature [2–4]. Several points merit further discussion.

Although some experimental models of autoimmunity rely solely on self-protein–peptide immunization for 'purely autoimmune' disease induction, it is usually accepted that, in infection-based diseases such as Chagas disease, the infectious agent is essential for the initiation of pathogenesis and that potential for further aggravation results from autoimmune responses. In fact, many mechanisms of inflammation other than autoimmunity – including direct cytolysis, antiparasitic immunity and microvascular spasm – have been demonstrated in humans or animals. The tremendous variability in the

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EDITORIAL
MALARIA LIVER STAGE CULTURE: *IN VITRO VERITAS*?

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Seven decades passed between Laveran's discovery of the blood stage malaria parasites in 1880 and the studies of Shortt, Garnham, and others that demonstrated the exo-erythrocytic phase of parasite development in the mammalian liver.[1,2] In part, this delay may be ascribed to Fritz Schaudinn's famous and erroneous "observation" in 1903 of the *Plasmodium vivax* sporozoite directly invading the red blood cell,[3] although as we now know the mammalian malaria parasites must develop in the liver for several days prior to initiating the erythrocytic phase of infection. Schaudinn's model held sway for decades despite the fact that no one could reproduce his findings.

Shortt and Garnham made their landmark observation in rodent malaria parasites,[1] as well as malaria parasites of humans and nonhuman primates,[2,4] but subsequent morphologic and biologic studies of the liver stage have primarily focused on rodent malaria parasites.[5] Inside hepatocytes, sporozoites transform into liver stage parasites, which are also called exo-erythrocytic forms (EEFs). Each liver stage trophozoite grows, undergoes multiple nuclear divisions as a schizont, and ultimately differentiates into tens of thousands of first-generation merozoites. These merozoites are released into the liver sinusoids where they infect red blood cells. Liver stage parasites cause no overt pathology and no detectable symptoms, and it is interesting that the parasite has selected an immunologically tolerogenic organ[6] as its bridgehead into the mammalian host.

Despite some progress in describing liver stage development, EEFs have remained frustratingly recalcitrant to reveal their biologic secrets during the nearly six decades since their discovery. Thus, liver stage cell biology and molecular biology of especially human malaria parasite liver stages are still in their infancy. Notwithstanding the fact that whole genome sequences are now available for a number of malaria parasite species and that modern systems biology tools can now analyze microarrays and high throughput proteomic studies that have been applied to all other life cycle stages of malaria parasites,[7] comprehensive gene and protein expression profiles of EEFs have yet to be established. Liver stage parasites may be the most promising target for a vaccine that completely prevents infection. Complete protection has been repeatedly demonstrated by vaccination studies using irradiation-attenuated live sporozoites, which induce complete sterile protection against challenge.[8] The protective mechanisms appear to mainly act against the liver stage. Therefore, elucidating the antigenic composition of liver stage parasites is critically important.

Why are human malaria liver stages so hard to study? First, they are difficult to find, the parasitologic equivalent of the "needle in the haystack." A typical mosquito may inoculate dozens of sporozoites into a host that if uniformly successful at invading hepatocytes develop into one liver stage each. That results in a few dozen liver stage parasites in a three-pound organ! Equally difficult to find are human volunteers willing to undergo a liver wedge biopsy, and for this reason C. H. Howard, who underwent open surgery and biopsy of the liver after receiving hundreds of infective mosquito bites for the purpose of discovering the liver stage parasite, may deserve equal billing with Shortt and others in the discovery of the exo-erythrocytic form of *P. falciparum*.[2]

Rodent malaria models have the invaluable advantage that *in vivo* infected livers are directly accessible for analysis, but even here the paucity of liver stage material and the technical challenge to isolate infected hepatocytes have made it difficult to study liver stages *in vivo*. *In vitro* systems such as the human hepatoma cell line HepG2 that supports development of *P. berghei* rodent malaria parasites[9] have been a great advance, but unfortunately this cell line and others do not support *P. falciparum* liver stage development with the exception of one cell line that supports it with low efficiency.[10] Thus, to date the only reliable *in vitro* system available for the culture of *P. falciparum* are primary human hepatocytes.[11]

In this issue of the journal, Sattabongkot and others describe a new hepatocyte line, HC-04, which was derived from normal human hepatocytes.[12] By painstakingly assessing sporozoite invasion and liver stage development, these investigators isolated a continuous cell line that sufficiently supports liver stage development of *P. falciparum* and *P. vivax* to yield merozoites that can initiate the erythrocytic phase. Because HC-04 supports liver stage development of the two most prevalent human malaria parasites, it will allow detailed comparative analysis of their liver stage biology. This may, for example, shed light on the differential growth regulation that must occur in dormant liver stages known as hypnozoites, which are responsible for the relapses of *P. vivax* malaria. Development of both parasite species appeared asynchronous in HC-04, but the timing of first merozoite release and thus the time to reach liver stage maturity corresponded approximately to the duration of *in vivo* development. A main advantage of this cell line over previously tested cell lines and primary human hepatocyte cultures is the greatly improved infection rates that are critical for using *in vitro* liver stage cultures in downstream experimental applications.

Sattabongkot and others have taken a great step forward with HC-04, but improvements on the HC-04 system will be needed to realize the full potential of liver stage cultures. The generation of clonal HC-04 lines and selection of clones with superior sporozoite infection and liver stage development rates is but one avenue for improvement. The present advance lays the ground for a routine, standardized, high-efficiency *in vitro* culture of the liver stages of human malaria parasites to be developed. Such a culture system will acceler-

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ate progress in important areas of malaria research, for example the identification of protective liver stage antigens, screening of novel drugs that act against liver stage parasites, and safety testing of live-attenuated whole sporozoite vaccines.

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Artemisinin combination therapies

Global mortality from *Plasmodium falciparum* malaria has always been enormous, and has increased in recent decades as chloroquine-resistant parasites spread worldwide. Alarms were raised further when parasites developed resistance to sulfadoxine-pyrimethamine soon after it was introduced to replace chloroquine in many areas. To protect the few remaining drugs in the malaria armamentarium, combination therapy is now touted, particularly combinations that include artemisinin derivatives, widely known as artemisinin combination therapies (ACTs).

Artemisinins are appealing. They work quickly, appear safe and well-tolerated, and might decrease malaria transmission by inactivating or killing gametocytes (the parasite form that transmits to mosquitoes). Several ACTs exist that differ either in the artemisinin derivative or its partner drug. Choosing the right ACT is very important for Ministries of Health. However, little is known about the comparative efficacy or sustainability of the different ACTs.

In today's *Lancet*, Frank Smithuis and colleagues compare two ACTs, dihydroartemisinin-piperaquine and artesunate-mefloquine, for treating falciparum malaria.[1] They found that both combinations were highly efficacious and effective in Burma. Artesunate-mefloquine was recently introduced as first-line treatment in Burma, but the ineffective drug chloroquine is still widely used because of the high cost of artesunate-mefloquine. The researchers note that dihydroartemisinin-piperaquine (about US$1·50 per adult treatment) is much cheaper than artesunate-mefloquine (about $3). Should everyone be switching to dihydroartemisinin-piperaquine?

Most countries in Africa, where antimalarial resistance has been an unmitigated disaster, are switching to artemether-lumefantrine. Until recently, this was the only coformulated ACT. Coformulation prevents inappropriate drug use that can occur when different pills are administered together. Furthermore, South Africa rapidly contained a deadly malaria epidemic in KwaZulu-Natal by using artemether-lumefantrine with other antimalarial measures in 2000–01.[2]

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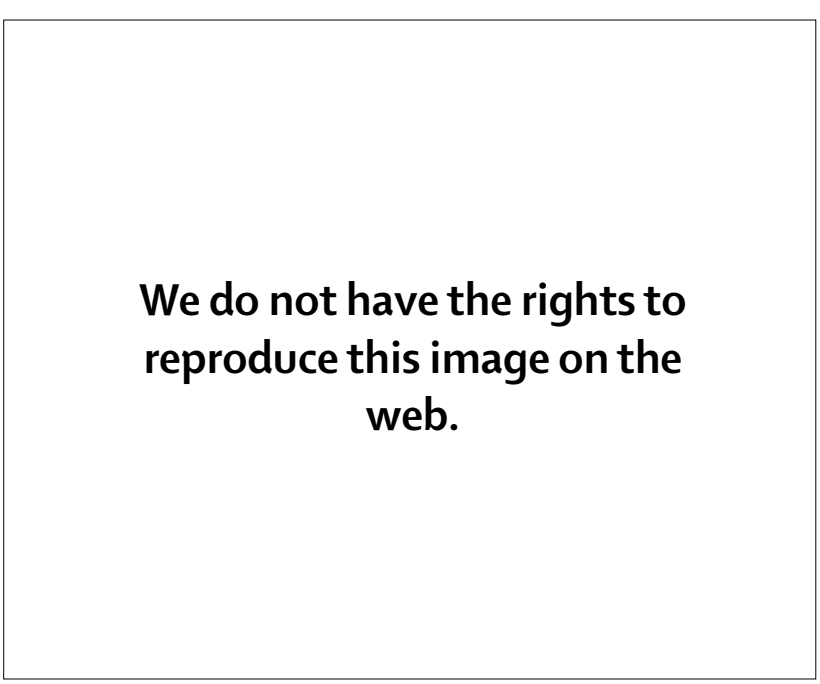
However, the promise of artemether-lumefantrine remains unfulfilled. There has not been enough drug to meet surging demand, and because of cost ACTs remain first-line therapy as policy but not as practice in many countries. Furthermore, reports of treatment failure emerged soon after artemether-lumefantrine was introduced in Zanzibar, with genetic evidence for selection of lumefantrine-resistant parasites.[3,4]



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Scanning electron micrograph of red blood cells and *Plasmodium falciparum*

Generally, ACTs do not work well when parasites are resistant to the partner drugs.[5,6] In southeast Asia, artesunate-mefloquine seemed an exception to the rule. The efficacy of that combination appeared to be stable over time despite high-level resistance to mefloquine when it was introduced.[7] This hopeful trend might have reversed recently, with reports of 30% treatment failures at one site in Thailand.[8] If resistance to artesunate-mefloquine spreads further, Thailand will have to change its first-line therapy. Other countries must also decide which drug combination is the best choice for their citizens. But good decision-making requires more studies like the one by Smithuis and colleagues, and from more malaria-transmission settings, including Africa, where some comparative ACT trials are now being reported.[9,10] Good choices require more ACTs, that are coformulated with effective partner drugs, and made in factories prequalified by WHO for good manufacturing practices.

ACTs would also benefit by being compared with non-ACT combinations. In Uganda, malaria was less common in children treated with sulfadoxine-pyrimethamine plus amodiaquine versus sulfadoxine-pyrimethamine plus artesunate.[11] Apparently, the short half-life of artesunate rendered the children susceptible to new infections soon after treatment. A mother of a sick child getting repeated malaria infections will not differentiate a recrudescence from a new infection—her child is sick in either case. If the switch to ACTs in areas of high transmission in Africa is accompanied by an overall increase in the number of children with malaria, the present environment of hopeful anticipation could easily turn hostile and impede policy uptake.

Smithuis and colleagues' study produced findings that policymakers elsewhere will note. First, both drug combinations were effective and efficacious, suggesting that compliance was high even when therapy was not taken at home. Second, mefloquine tolerability appeared reasonably good, even with the high dose used in the study. Dizziness (in about half) and nausea (in a quarter) were common, but the frequency of vomiting (about 2%) was low and similar in the two groups. Other neuropsychiatric sequelae were not reported, but should be looked for in future studies.

Third, dihydroartemisinin-piperaquine was significantly less effective than artesunate-mefloquine for preventing gametocytaemia. Is this an important handicap? Most infected Africans are asymptomatic because of immunity and do not seek treatment. These individuals represent a large reservoir for continued transmission of malaria that will dilute any effect of artemisinins. However, if the sustainability of artesunate-mefloquine in Thailand has been due to its gametocyticidal activity (preventing the spread of resistant parasites that recrudesce in treated individuals), this activity may be an important criterion for selection.

The availability of effective antimalarial drugs is a life-or-death matter for millions of people. ACTs offer hope, but we do not yet know which drug combinations are most effective, or most sustainable, in different populations. That knowledge requires many more studies and continued surveillance for resistance after use of ACTs is implemented.

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Climate change and health

As editors of *Public Health*, we often publish on topics that are of interest to a wider readership than the traditional public-health community, especially because we have become increasingly aware that the public-health agenda is now a central concern of a wider range of professionals. Among this group are those people whose work clearly affects the health of populations—eg, those who are involved in the sustainable development movement. At last year's UK Public Health Association's Annual Forum, Porritt[1] showed that public health is integral to the sustainable development agenda, with what he called "blindingly obvious" examples. For instance, hospital food is generally bad but "good" hospital food is good for patients; so more effective hospital catering and procurement policies would result in more nutritious hospital food. Such policies would be good for the care of patients, and benefit staff and visitors. The UK's National Health Service could use its power as one of the country's largest food purchasers to encourage local and sustainable food production and to promote sound agricultural practices. Porritt extended the hand of partnership of the sustainable development movement to the public-health community, which should be grasped enthusiastically by policymakers and activists alike.[2]

We were delighted when Richard Horton proposed that *The Lancet* and *Public Health* co-publish Andy Haines' 2005 Harben Lecture on climate change and human health.[3,4] This is not the first time *The Lancet* has shown interest in this subject.[5] The Harben Lecture is an annual event hosted by the Royal Institute of Public Health. Over its long history, the lecture has attracted many expert and influential speakers, and Haines' topic is one of the most stimulating and thought-provoking, not to mention timely.

By publishing the Harben Lecture on climate change, we provide the loudest possible voice for one of the greatest and global public-health issues. Next month, *Public Health* will be including the paper alongside other international contributions that highlight the significance of these related environmental issues. Other issues to be addressed in a mini-symposium on the impacts of the natural and built environments on health include: sustainable development in the National Health Service, governance of public utilities for a sustainable future, and maturation of the concept of sustainable development in a link between China and Europe. This mini-symposium will also include consideration of the somewhat overlooked role of health services everywhere in creating sustainable solutions.

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Effects of gender, parity and sequence variation on seroreactivity to candidate pregnancy malaria vaccine antigens

Running title: Antibody responses to VAR1CSA and VAR2CSA

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Abstract

Background: Plasmodium falciparum–infected erythrocytes (IE) adhere to chondroitin sulfate A (CSA) to sequester in the human placenta, and placental malaria (PM) is associated with disease and death of both mother and child. A PM vaccine appears feasible, because women become protected as they develop antibodies against placental IE. Two IE surface molecules, VAR1CSA and VAR2CSA, bind CSA *in vitro*, and are potential vaccine candidates.

Methods: We expressed all domains of VAR1CSA and VAR2CSA as mammalian cell surface proteins using a novel approach that allows rapid purification, immobilization and quantification of target antigen. We measured reactivity of sera from East Africa to all domains, and examined effects of host gender and parity, as well as parasite antigenic variation.

Results: The reactivity to all VAR2CSA domains was higher with sera from multigravid females than primigravid females or males. Conversely, reactivity to VAR1CSA domains was consistently higher with sera from males than gravid females. Seroreactivity was strongly influenced by antigenic variation of VAR2CSA DBL domains.

Conclusions: Women acquire antibodies to VAR2CSA over successive pregnancies, but lose reactivity to VAR1CSA. Serum reactivity to VAR2CSA is variant-specific, and future studies should examine the degree to which functional antibodies such as binding-inhibition antibodies are variant-specific.

Key words: pregnancy malaria, PfEMP1, VAR1CSA, VAR2CSA, quantitative protein array, immunoprofiling.

Introduction

Plasmodium falciparum parasites sequester in the human placenta [1], and placental malaria is associated with disease and death of both mother and child [2-5]. Previous studies identified chondroitin sulfate A (CSA) as a major receptor molecule for sequestration of infected erythrocytes (IE) in the placenta [6]. Malaria parasites variably express antigens on the IE surface that bind a variety of endothelial receptors [7, 8], including CSA. PfEMP1 is a variant surface antigen (VSA) family encoded by about 60 *var* genes per malaria parasite genome [9], and these have been implicated in a number of binding interactions. The sequences of *var* genes vary substantially within and between genomes. PfEMP1 forms are expressed in a mutually exclusive manner [10], creating extensive antigenic variation and the potential for multiple adhesion profiles. This variation is a major obstacle to the development of a PfEMP1-based anti-malarial vaccine.

Resistance to pregnancy malaria increases over successive pregnancies [3] as women acquire antibodies against placental parasites. Sera from immune multigravidae, even from distant geographical regions, but not males, can inhibit binding of placental IE to CSA [11], and this serum activity is related to protection from infection and disease during pregnancy [12, 13]. Two PfEMP1 molecules, VAR1CSA and VAR2CSA, have been implicated in pregnancy malaria and are potential vaccine candidates (reviewed in [14]). Both are large molecules of more than 350 kDa with 7 and 6 distinct Duffy binding-like (DBL) domains, respectively, and each is extensively cross-linked by disulfide bonds.

To study the involvement of these molecules in protective immunity, we expressed all domains of VAR1CSA and VAR2CSA on the surface of mammalian cells as GFP fusion proteins using a novel vector that allowed rapid purification, immobilization, and quantification of antigen. We prepared arrays of individual VAR1CSA and VAR2CSA domains from laboratory strains and field isolates, and tested their immune reactivity with sera from East African donors to determine the effects of host gender and parity, as well as parasite antigenic variation, on antibody recognition.

Materials and Methods

Vector for expression of malaria antigens on the surface of mammalian cells

The DNA sequence encoding Enhanced Green Fluorescent Protein (EGFP) was excised from pEGFP-N1 (Clontech) by XhoI/NotI digestion. Sequence encoding trans-membrane and cytoplasmic (TMC) domains of the rat surface receptor megalin [15] was RT-PCR-amplified using forward and reverse primers with EcoRI and XhoI sites at their 5'-ends, respectively (forward 5'-tttgaattcctccagggacgacaatggctgtt-3', reverse 5'-tttctcgagtacgtcggatcttctttaacgag-3'), then digested with EcoRI and XhoI. Plasmid vector pSecTag2C (Invitrogen) was digested with BamHI and EcoRI, then ligated to a double-stranded (ds) oligonucleotide adaptor (AdEx) with a multicloning site created by annealing two single-stranded (ss) oligonucleotides: 5'-gatccttaagtccggaggcgcctctagacttaacgg-3' and 5'-aattccgttaactctagaggcgcctccggacttaag-3'. The resulting vector was digested with EcoRI and XhoI then ligated to the megalin TMC fragment described above. This construct, in turn, was digested with XhoI and Bsp120I and ligated to the EGFP fragment. The resulting vector was digested with XhoI and AgeI to remove double digestion

sites, then ligated to a ds oligonucleotide adaptor (created by annealing two ss oligonucleotides: 5'-tcgagctgaagcttcgaatcctgcagtcgacggtaccgcgggcccgggaccca-3' and 5'-ccggtgggtcccgggcccgcggtaccgtcgactgcaggattcgaagcttcagc-3') that introduced point mutations to eliminate unwanted restriction sites. The resulting vector, called pAdEx, was used to clone and express the *P. falciparum* antigens described in this work (Figure 1). The integrity of the construct was verified by restriction digestion and sequencing.

Cloning malaria antigen genes into the pAdEx vector

DNA encoding each antigen was amplified by PCR from strains FCR3 and 3D7 *P. falciparum* genomic DNA, or from cloned 661 cDNA (see below), using primer pairs with appropriate restriction enzyme sites (Table 1). After PCR, amplified DNA fragments and pAdEx vector were digested, ligated, and cloned. The integrity of each construct was verified by sequencing.

Cloning and sequencing var2csa from placental parasite sample 661

The clinical placental parasite sample 661 was obtained from placental intervillous blood following delivery at the Muheza Designated District Hospital, Muheza, Tanzania, from a woman participating in the MOMS Project (described in [16]). Parasite samples were stored in RNALater (Ambion) at -20^{o}C. RNA was isolated according to the manufacturer's instructions using Trizol (Invitrogen). Purified RNA was treated with DNA-free reagent (Ambion) to remove genomic DNA. RNA was then reverse transcribed using Superscript III and random hexamers (Invitrogen) for 2 hours at 42^{o}C. DBL6 primers AAGAACATTGTTCTAAATGTC (Forward) and TGTAAATATTGTTCAATAAAATCC (Reverse) were designed by aligning PFL0030c

sequences from strains 3D7 and ITG (Accession Number AY372123.1) to identify conserved sequences that flank the DBL6 domain. The PCR product was cloned into pCR2.1 TOPO TA Cloning System and sequenced in both directions.

Preparation of Quantitative Protein Arrays with malaria antigens

COS-7 cells (50 to 70 % confluent) were transfected with various constructs using Fugene transfection reagent (Roche) according to the manufacturer's protocol. Cells from each 150 mm^2 flask were lysed 48 hours post-transfection (transfection efficiency >80%) with 5 ml of CellLytic reagent (Sigma). Recombinant products were confirmed on Western blots with anti-GFP monoclonal antibody (Clontech, 1:500 dilution), followed by HRP-conjugated anti-mouse IgG (Sigma, 1:1000 dilution). Concentrations of fusion proteins (in relative units) were measured by GFP fluorescence using the Fluoroskan Ascent FL fluorometer/luminometer (Thermo Labsystems, Waltham, MA), then equalized by dilution with lysate of non-transfected cells (lysate K). 100 µl diluted lysate was added to each well of 384-well white plates coated with anti-GFP antibody (Pierce) and incubated overnight at 4^oC. Undiluted lysate K was used as a control for non-specific background fluorescence and chemiluminescence. Lysate prepared from cells transfected with control construct (pAdEx vector alone without malaria antigen fusion partner) was used as a negative control in each assay. After washing with Washing Buffer (WaB) (PBS plus 0.05% Tween-20), plates were ready for immunoprofiling experiments with human serum samples.

Validation of expressed MSP-1 antigen by structure-sensitive monoclonal antibody

Recombinant MSP-1_{19} or control construct product AdEx were immobilized in anti-GFP plates as described above, then incubated with mouse monoclonal antibody mAb 12.10 reactive only to the properly folded structure of MSP-1 [17] (generously provided by Dr. J.A. Lyon, WRAIR) at 1:5000 dilution, followed by HRP-conjugated anti-mouse IgG (Sigma, 1:1000 dilution). Reactivity signals were obtained (as relative luminescence units, RLU) using 100 µl of ECL chemiluminescence substrate (Amersham Biosciences) per well and Fluoroscan luminometer.

Serum samples

Human sera used in these studies were collected from East African donors under protocols approved by relevant ethical review committees. Study participants provided written informed consent before donating samples, and included adult males and multigravid women from Kenya [18, 19] and multigravid and primigravid women from Tanzania [20]. Briefly, 18 – 45 yo. multigravidae and 18 – 50 yo. males (median ages 28 and 29 years, respectively, P = 0.62) from Kenya, and 18 – 45 yo. gravidae from Tanzania, were included in the study. Sera from pregnant women were collected at the time of delivery and tested individually. The number of sera used in each experiment is indicated in the corresponding figure legend. Sera from 10 randomly selected non-immune donors in the US were separated from whole blood obtained from commercial sources (Valley Biomedical) and used in a pool as a negative control.

Immunoprofiling study of human sera on malaria antigens

All sera were pre-incubated for at least 24 hours at 4^{o}C with an equal volume of 10 mg/ml goat IgG to eliminate non-specific reactivity against goat anti-GFP IgG bound to the wells of 384-well

plates. The pre-incubated sera were further diluted 1:100 with Superblock (Pierce) and incubated with the antigen array for 2 hours at room temperature (RT). After 3 washes with WaB, plates were incubated with Donkey anti-human IgG (H+L) affinity-purified antibody conjugated to HRP (Jackson Immunoresearch) diluted 1:1000 in Superblock. After 1 hour at RT, wells were washed, then 100 µl of ECL chemiluminescence substrate (Amersham Biosciences) was added per well, and chemiluminescence and fluorescence signals were measured. The use of the chemiluminescence substrate does not affect the fluorescence measurement.

Chemiluminescence signal reflects immune reactivity and fluorescence signal reflects the amount of immobilized antigen–GFP fusion proteins. Fluorescence signal was corrected by subtraction of background values measured in lysate K wells, then the immunoreactivity signal (chemiluminescence) was normalized to the amount of immobilized antigen (fluorescence) in each well. Average reactivity was calculated for duplicate wells, and a final specific immunoreactivity (in arbitrary units, AU) was calculated by subtraction of Control Value (defined as either the average reactivity of the same serum to control construct plus 3 SD, or the reactivity of pooled non-immune sera to the same antigen plus 3 SD, whichever was higher). Correlations were analyzed using Spearman rank test. Differences between group reactivities were tested for significance by Mann-Whitney test. P values less than 0.05 were considered significant. GraphPad Prizm software was used for all statistical analyses.

Results and Discussion

Features and performance of Quantitative Protein Arrays

Heterologous expression of malaria surface antigens is known to be difficult, in part due to their high AT content (up to 80%) and their highly conformational cysteine-rich structure. An expression system that provides a trans-membrane protein trafficking pathway and cell-surface presentation may significantly improve the co-translational folding of PfEMP1 surface molecules, in which each domain contains 6-9 disulfide bonds. We engineered a pAdEx vector encoding a hybrid receptor with a signal peptide (from immunoglobulin kappa chain), an extracellular domain, and individual trans-membrane and cytoplasmic domains (both from the single-spanning transmembrane receptor megalin) (Figure 1). The cytoplasmic domain has signals that direct this protein to the plasma membrane surface. In addition, the GFP-reporter protein is fused to the cytoplasmic domain and reports protein expression levels, which can be quantified. The multicloning site allows simple and rapid preparation of different constructs that express *Plasmodium* antigen extracellular domains on the surface of mammalian cells.

Using this construct, we expressed several DBL domains from *var1csa* and *var2csa* genes in addition to other *P. falciparum* antigens (AMA-1 and MSP-1 19 kDa carboxy-terminal fragment) as GFP fusion proteins (Figure 2). All antigens were successfully expressed using the native malaria coding sequence. CIDR-alpha domains always follow DBL-alpha domains, and may act as a single functional domain [9], so *var1csa* DBL1-alpha domain was expressed together with CIDR1-alpha domain. For negative control wells, we used a GFP fusion protein (AdEx) containing an irrelevant extracellular domain of 37 amino acids that resulted from the translation of the multicloning site in the pAdEx DNA construct.

The integrity of fusion proteins was tested by Western blotting with anti-GFP antibodies (Figure 3A). Recombinant proteins demonstrated the expected molecular weight and produced green fluorescence in cells as well as in cell lysates. Fluorescence was preserved after immobilization of fusion proteins in 384-well plates. GFP fluorescence has been shown to be a good indicator of properly folded membrane proteins when GFP is fused to the cytoplasmic tail [21]. We also tested reactivity of the disulfide-rich MSP-1 19kDa fusion protein using conformation-dependent mAb 12.10 [17], which readily recognized the antigen (Figure 3B), confirming correct folding.

Malaria antigens were organized into arrays using a single-step procedure in 384-well plates. The GFP fusion partner has a number of advantages. First, the tag can be used for immobilization and purification of antigens in a single simple step. Second, the GFP allows the amount of antigen in each lysate to be measured and equalized, thereby reducing variance. Third, the immunoreactivity of sera (measured by chemiluminescence) can be normalized to the amount of antigen (measured simultaneously by GFP fluorescence) in each well, which further reduces variance.

Seroreactivity to irrelevant antigen is common in malaria endemic areas

As observed in earlier studies [22, 23], we found that immune sera from malaria endemic regions frequently react to completely irrelevant proteins (data not shown), and this non-specific reactivity corresponds to an elevated reactivity to malaria antigens. In contrast, non-immune sera (NIS) from individuals living in non-endemic areas have low non-specific reactivity. For this reason, NIS control may not be adequate to demonstrate specific reactivity of test sera in malaria

seroepidemiology studies, since this approach may falsely identify those sera with high levels of non-specific reactivity as positive. The use of the control construct provides the means to quantify and therefore correct for non-specific reactivity of each construct in each individual serum sample.

Seroreactivity to VAR1CSA and VAR2CSA has a dichotomous pattern related to gender

We measured seroreactivity of East African and non-immune individuals to domains of VAR1CSA and VAR2CSA expressed as GFP fusion proteins. AMA-1 was used as positive control because it is known to react strongly to the majority of sera from malaria endemic regions [24]. As expected, immune sera uniformly reacted at high levels to relatively conserved AMA-1, and seroreactivity did not differ between males and multigravid females (inset in Figure 4A, median for male sera was 5289 AU, median for multigravid sera was 5872 AU, P=0.46, N=44 and 52).

Immune responses to PfEMP1 domains were substantially lower and more variable (Figure 4) compared to AMA-1 responses. Two VAR1CSA domains, DBL6β and DBL7ε, and one VAR2CSA domain, DBL2X, were non-reactive or minimally reactive in our screens. Non-reactivity of VAR2CSA DBL2X was likely due to rapid degradation of this fusion protein during and after cell lysate preparation, detected by Western blotting (data not shown). The reason for non-reactivity of VAR1CSA DBL6 and DBL7 is not clear, since the proteins were stable. The results suggest weak host immunoreactivity against these domains, but we cannot exclude that the proteins were incorrectly folded in a way that disrupted or masked structural epitopes.

The variable response to VAR1CSA and VAR2CSA was related to gender of the serum donors. Consistent with earlier studies from West Africa [25-27], the reactivity of all VAR2CSA domains (other than DBL-2X) was significantly higher with sera from multigravidae than sera from males (Figure 4). Out of 54 Kenyan multigravid sera tested in this experiment, 10 samples were from women with pregnancy malaria (PM). Antibody levels were not significantly different (data not shown) in women with PM versus those without PM, possibly reflecting the brief duration of infection in multigravid women [3, 28, 29] or that antibody levels may be maximal in this parity group by the time of delivery. Previous studies in West Africa that examined three DBL domains (DBL1, DBL5 and DBL6) of VAR2CSA expressed in baculovirus [25, 26] found that seroreactivity was significantly higher in multigravidae than males to domains 5 and 6 but not domain 1. The increased reactivity of sera from multigravid women in East Africa against all VAR2CSA domains supports the idea that this PfEMP1 molecule is preferentially expressed by pregnancy malaria parasites, and that women acquire antibodies against this protein as they become protected. Reactivity to DBL1X domain correlated significantly to reactivity against three other domains (DBL3, Spearman $r = 0.29$, $P = 0.04$; DBL5, $r = 0.41$, $P = 0.003$; DBL6, $r = 0.48$, $P = 0.0003$) but not against AMA-1 antigen ($r = 0.07$, $P = 0.6$), suggesting that immunity to different VAR2CSA domains is acquired concordantly.

The pattern of reactivity to VAR1CSA versus VAR2CSA domains diverged markedly, and was consistent against all tested domains (Figure 4). Sera from multigravid women reacted more strongly to VAR2CSA domains, while sera from men reacted more strongly to VAR1CSA domains. The increased antibody levels of males versus multigravidae were statistically significant

for two VAR1CSA domains (DBL1α and DBL5γ). Multigravidae and men had similar reactivity to AMA-1 (see above) and MSP-1 (data not shown), indicating that multigravidae specifically lose reactivity to VAR1CSA. Our studies of AMA-1 and MSP-1_{19} are similar to numerous earlier studies, which found that seroreactivity to various non-PfEMP1 malaria antigens did not vary with the pregnancy status or parity of sample donors (reviewed in [30]).

The dichotomous pattern of reactivity of men and multigravidae may be explained by mutually exclusive expression of *var* genes in *P. falciparum* [10]. Placental malaria is caused by CSA-binding parasites [6] that preferentially express *var2csa* [31], and peripheral parasites in pregnant women have features similar to placental parasites [16, 32]. Thus, the upregulation of *var2csa* in placental parasites may be accompanied by a downregulation of other commonly expressed *var* genes, such as *var1csa*. Antibodies against VAR1CSA domains may therefore diminish in pregnant women, who would receive antigenic stimulation by VAR2CSA but not VAR1CSA during episodes of pregnancy malaria.

Seroreactivity to VAR2CSA increases with gravidity

We compared sera from multigravid (all 32 without PM) versus primigravid (8 with PM and 24 without PM) women in Tanzania for immunoreactivity to VAR2CSA domains. As was previously observed [25, 26] (see below), VAR2CSA seroreactivity increased with the number of pregnancies (Figure 5), and, consequently, with protection status. Differences in seroreactivity between gravidity groups were statistically significant for 3 domains (DBL1, DBL3 and DBL6). These differences remained significant in analyses that only included sera from women without PM. In

earlier studies, seroreactivity of the DBL5 domain [25] and 3 VAR2CSA domains (DBL1, DBL5 and DBL6) [26] correlated significantly with gravidity, but the levels of seroreactivity were not significantly different between gravidity groups. Interestingly, antibody levels to VAR2CSA domains 1, 3 and 6 were significantly higher ($p<0.05$ for all comparisons, data not shown) among first-time Tanzanian mothers with PM versus those without PM in our study, suggesting specific responses to the antigen during pregnancy malaria. Separate studies will need to examine whether the antibodies produced by first time mothers during malaria episodes have functional activity.

Similar studies were previously undertaken in West Africa with two VAR1CSA DBL domains (DBL1 and DBL2) expressed in *E.coli* [33] and varying numbers (two [25], three [26], or six [27]) of VAR2CSA domains expressed in the baculovirus system. In those studies, male and female serum reactivity against VAR1CSA domains did not differ significantly. Of note, *E.coli*-expressed DBL antigens may not recreate the extensive disulfide bonds and folds of the native protein [14], and expression of the DBL1 domain separate from the CIDR domain may disrupt a single functional domain and alter its conformation. In our studies, VAR1CSA domains were expressed on the surface of mammalian cells in order to better reproduce the native structure of these complex antigens, which may allow better discrimination of seroreactivity differences. The earlier studies of VAR2CSA, which we discussed in detail above, generally observed a gender-specific and parity-specific pattern of reactivity; supporting the idea that VAR2CSA is preferentially expressed by placental parasites and is targeted by antibodies that correlate with immunity.

In our work, we expanded on these prior studies to incorporate all domains from each PfEMP1 protein, expressed each one in a mammalian system to increase the probability of correct folding, and studied them together using sera from a distinct geographical region, East Africa. These studies show for the first time that all immunoreactive VAR1CSA domains were recognized at higher levels by sera from males versus multigravid women, and that reactivity was highest against the first VAR1CSA domain (DBL1α plus CIDR1α). We also demonstrated that all immunoreactive VAR2CSA domains react more strongly to sera from multigravid women, and confirm that this reactivity is parity-specific.

Reactivity to VAR2CSA is variant-specific

We compared reactivity of immune sera to variant forms of domain DBL6ε representing laboratory isolate 3D7 and fresh placental parasite 0661. These variant forms have a high level of homology throughout most of their sequence (Figure 6A). Individual sera from multigravid women varied substantially in their reactivity to variant forms of DBL6ε (Figure 6B). Antigenic variation in this domain is limited to areas comprising about 30% of the domain sequence, primarily in the loops between helices alpha 2 and alpha 4, and between alpha 5 and alpha 7 [34]. Since the remainder of the domain is largely conserved, and the immune response against these two homologous domains is significantly different, we speculate that the immune response is predominantly directed toward regions of sequence variability including the loops. This may also indicate that the most conserved parts of the domain are poorly immunogenic. We also saw a similar pattern of differential reactivity with VAR2CSA DBL1X domains (identity was about 80% between variants, data not shown).

Previous work [26] demonstrated no relationship between serum levels of anti-3D7 VAR2CSA antibodies and anti-CSA-adhesion antibodies in 4 out of 6 placental isolates. This may have resulted from VAR2CSA sequence variation between placental samples as the authors suggested, or may indicate that functional antibodies are a minor subset of total antibody. Our results on differential reactivity of laboratory isolate (3D7) versus placental parasite (661) DBL domains do not confirm one or the other of these possibilities. If the former possibility is correct, then protective immunity in multigravidae may reflect the acquisition of antibodies against the VAR2CSA variants present in a community. A globally-related pool of polymorphisms accounts for sequence variation in VAR2CSA [35], and therefore a limited number of variants may be adequate to elicit broadly reactive antibodies. Such a vaccine may be able to target only the loop regions, which could significantly simplify the task of developing a vaccine

Future studies will need to identify the malaria antigen, domain or domain variant(s) and fragment(s) that are specifically targeted by protective antibodies, and that elicit broadly reactive antibodies. This information could provide the basis for a pregnancy malaria vaccine.

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Figure legends

Figure 1. Hybrid protein for expression of *P. falciparum* antigens on the surface of mammalian cells.

Figure 2. *P. falciparum* protein domains expressed and used for seroreactivity studies.

Indicated domains were expressed as GFP-fusion proteins in COS-7 cells and immobilized individually for antigen arrays. As a positive control, AMA-1-GFP fusion protein was used. TM – trans-membrane domain, Cyt – cytoplasmic domain.

Figure 3. Characterization of malaria antigens cloned as GFP-fusion proteins in pAdEx vector and expressed in COS-7 cells.

A. Western blot of expressed antigens with monoclonal anti-GFP antibody. 1 – DBL3γ from VAR1CSA, predicted molecular weight (MW) is 85 kDa; 2 – control construct mini-megalin with extracellular domain containing first ligand-binding domain of rat receptor megalin (nucleotides 1-1882) [15], predicted MW is 106 kDa; 3 – MSP-1 19 kDa fragment, predicted MW is 70 kDa. MW – molecular weight markers.

B. Interaction of structure-sensitive anti-MSP-1 monoclonal antibody 12.10 [17] with MSP-1 fusion protein. AdEx control construct (AdEx control) or MSP-1 (MSP-1) recombinant protein were immobilized in the wells of anti-GFP plates and tested for reactivity with monoclonal

antibody 12.10 followed by secondary anti-mouse HRP-conjugated antibody (mAb12.10) or with secondary antibody only (2nd Ab only). Signals were measured using chemiluminescent substrate. Bars represent average of 3 measurements, error bars are Standard Errors. RLU – relative luminescence units.

Figure 4. Sera from multigravidae preferentially react to VAR2CSA domains, while sera from males preferentially react to VAR1CSA domains.

Seroreactivity to VAR2CSA and VAR1CSA domains (after subtraction of the Control Value, see Materials and Methods) is indicated according to donor group. White bars – male sera; Gray bars – multigravid female sera. AU – Arbitrary Units. P values are results of two-tailed Mann-Whitney test (N = 52 multigravid and 44 male sera for the left graph, and 32 multigravid and 32 male sera for the right graph). The top, bottom and line through the middle of the box correspond to the 75^{th} percentile, 25^{th} percentile, and the 50 percentile (median), respectively. The whiskers indicate the 10^{th} and 90^{th} percentiles. Inset – reactivity of AMA-1 for both groups.

Figure 5. Serum reactivity to VAR2CSA domains increases with gravidity.

Seroreactivity to individual VAR2CSA domains is stratified by gravidity. White bars – primigravid female sera; Gray bars – multigravid female sera. AU – Arbitrary Units. P values are results of two-tailed Mann-Whitney test (N=32, 32 for each group). The top, bottom and

line through the middle of the box correspond to the 75th percentile, 25th percentile, and 50 percentile (median), respectively. The whiskers indicate the 10th and 90th percentiles.

Figure 6. Antigenic variation of VAR2CSA DBL6ε is restricted to the short regions, and substantially affects seroreactivity.

A. Comparison of 3D7 and 661 DBL6ε domain sequences. Sequence alignment was performed using CLUSTAL (-like) formatted alignment by MAFFT (v5.860) at GenomeNet web site (http://timpani.genome.ad.jp/~mafft/server/). Stars indicate conserved residues, colons and dots indicate more and less conservative substitutions. α2, α4, α5 and α7 are helical regions identifiable according to [34]. Blue boxes indicate regions of low homology. Yellow boxes indicate cysteines conserved between these two variants. **B**. Reactivity of 3D7 and 661 DBL6ε domains with five multigravid sera selected randomly from the set of sera used in previously described experiments. Black bars – 3D7 DBL6ε, white bars – 661 DBL6ε.

Table 1. PCR primers for amplification of antigen domains

Domain	Forward Primer	Reverse Primer
Var1csa DBL1α-CIDR (271-2280)	CCCGGATCCAGGATCATAAGGAACATACTAATTTACGG	CCCGAATTCCATTTTTAGTGGGTTGCGTGCCTCCACG
Var1csa DBL2β (2440-3402)	CCCCCTTAAGTCTAATCGTAATCTTGGTTTTTCAAATG	CCCGAATTCCAGACATTTGTGCTTGTTCATGTAATTC
Var1csa DBL3γ (3802-4698)	TTCGGATCCTTAAAGAAAACGATGGAAAGAAAC	TTTGAATTCCATAGTCTGTAACCATTACACCAATG
Var1csa DBL4ε (4855-5805)	CCCGGATCCAGGAAAATGACGACAAATATACTAACATT	CCCGAATTCCCTCGGAATATATTTTGTCTTTATTCTC
Var1csa DBL5γ (5968-7146)	CCCGGATCCAGGACGATGAACCAAAAGAAGTTGAAGG	CCCGAATTCCATCCTTATACTTTTTGCCATCTTTATC
Var1csa DBL6β (7594-8436)	CCCGGATCCAGGATAAATATATAGGAAGAAGAAACCC	CCCGAATTCCAGATTTCCATTTAAGAACAAAATTTTT
Var1csa DBL7ε (8761-9540)	CCCGGATCCAGAAGGAATTACAAACTTTTACCTTCTG	CCCGAATTCCTTTATTGTCTATATTACCTGAAGATTG
Var2csa-DBL1X (1-1347)	CCCCTCCGGAATGGATAAATCAAGTATTGCTAAC	CCCGAATTCCGATACATGTTTTATTCGACGACGG
Var2csa-DBL2X (1534-2586)	CCCCTCCGGATCTAGTTCTAATGGTAGTTGTAATAAC	CCCGAATTCCATTTGTAGTACTACTTGGGCCACAAT
Var2csa-DBL3X (3580-4557)	CCCGGATCCAGAAGGAAAATGAAAGTACCAATAATAAAA	CCCGAATTCCATCACTCGCAGATTTTCCTACATATTTA
Var2csa-DBL4ε (4708-5643)	CCCGGATCCAGGAGAAAAAAAATAATAAATCTCTTTG	CCCGAATTCCAGGTTCCATAATCATTGAATAATCTTT
Var2csa-DBL5ε (5944-7008)	CCCGGATCCAGTTAGATAGATGTTTTGACGACAAG	CCCGAATTCCTTTATTACAAATATAATCATTACC
Var2csa-DBL6ε (6973-7761)	CCCGGATCCAGGAGTATGATAAAGGTAATGATTATATTT	CCCGAATTCCTTTTTCTGCTTTGGTTTCTTTATAATTC
AMA-1 (70-1629)	CCCGGATCCAGGGACAGAATTATTGGGAACATCC	CCCTCTAGAATCATAAGTTGGTTTATGTTCAGG
MSP-1 19kDa CTD (4588-5160)	CCCGGATCCAGATTGTTGAAAAAGATGAAGCACATG	CCCGAATTCCAATGAAACTGTATAATATTAACATG
661-var2csa-DBL6ε	CCCGGATCCAGGAGTATGATAAAGGTAATGATTATATTT	CCCGAATTCCATTACCATTTTGGTTTTTAAATTTAGC

Numbers indicate nucleotide positions in the sequences of *var1csa* (FCR3 strain, accession number AJ133811), *var2csa* (3D7 strain, PFL0030c in PlasmoDB Database), *ama1* (3D7 strain, PF11_0344 in PlasmoDB), and *msp1* (3D7 strain, PFI1475w in PlasmoDB). Restriction enzyme sites are underlined. CTD – C-terminal domain

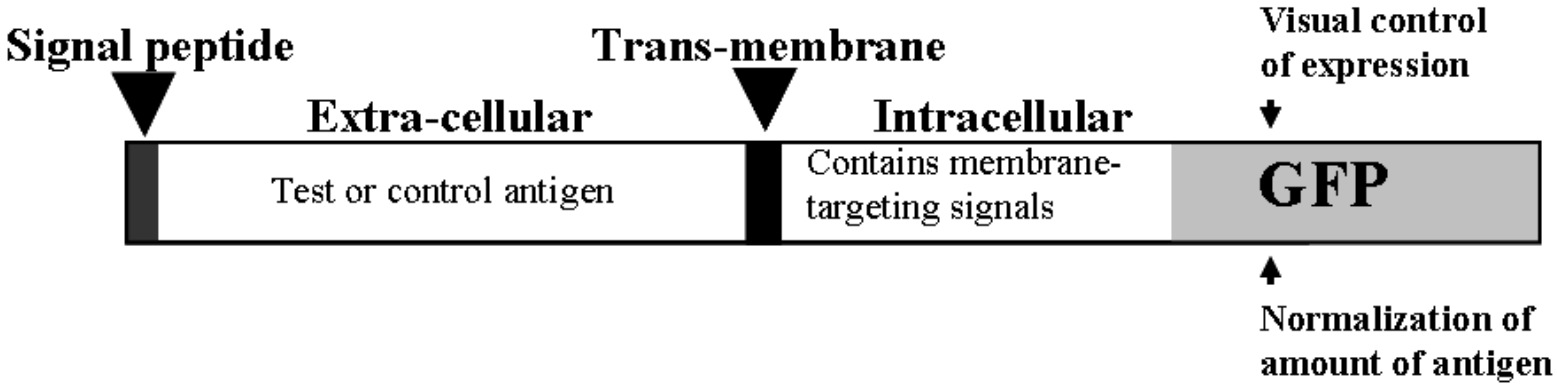

Signal peptide
Trans-membrane
Visual control of expression
Extra-cellular
Intracellular
Test or control antigen
Contains membrane-targeting signals
GFP
Normalization of amount of antigen

Figure 1

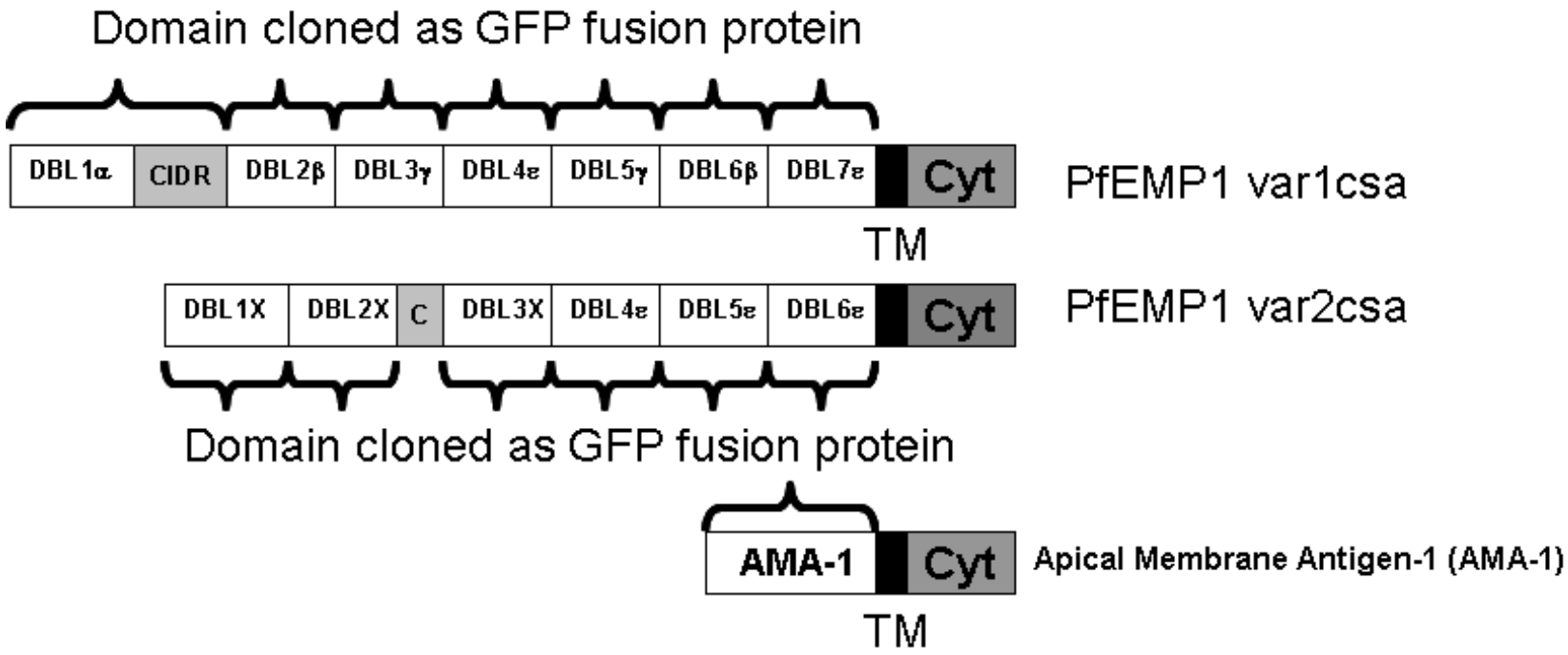

Domain cloned as GFP fusion protein
DBL1α
CIDR
DBL2β
DBL3γ
DBL4ε
DBL5γ
DBL6β
DBL7ε
Cyt
TM
PfEMP1 var1csa
DBL1X
DBL2X
C
DBL3X
DBL4ε
DBL5ε
DBL6ε
Cyt
PfEMP1 var2csa
Domain cloned as GFP fusion protein
AMA-1
Cyt
Apical Membrane Antigen-1 (AMA-1)
TM

Figure 2

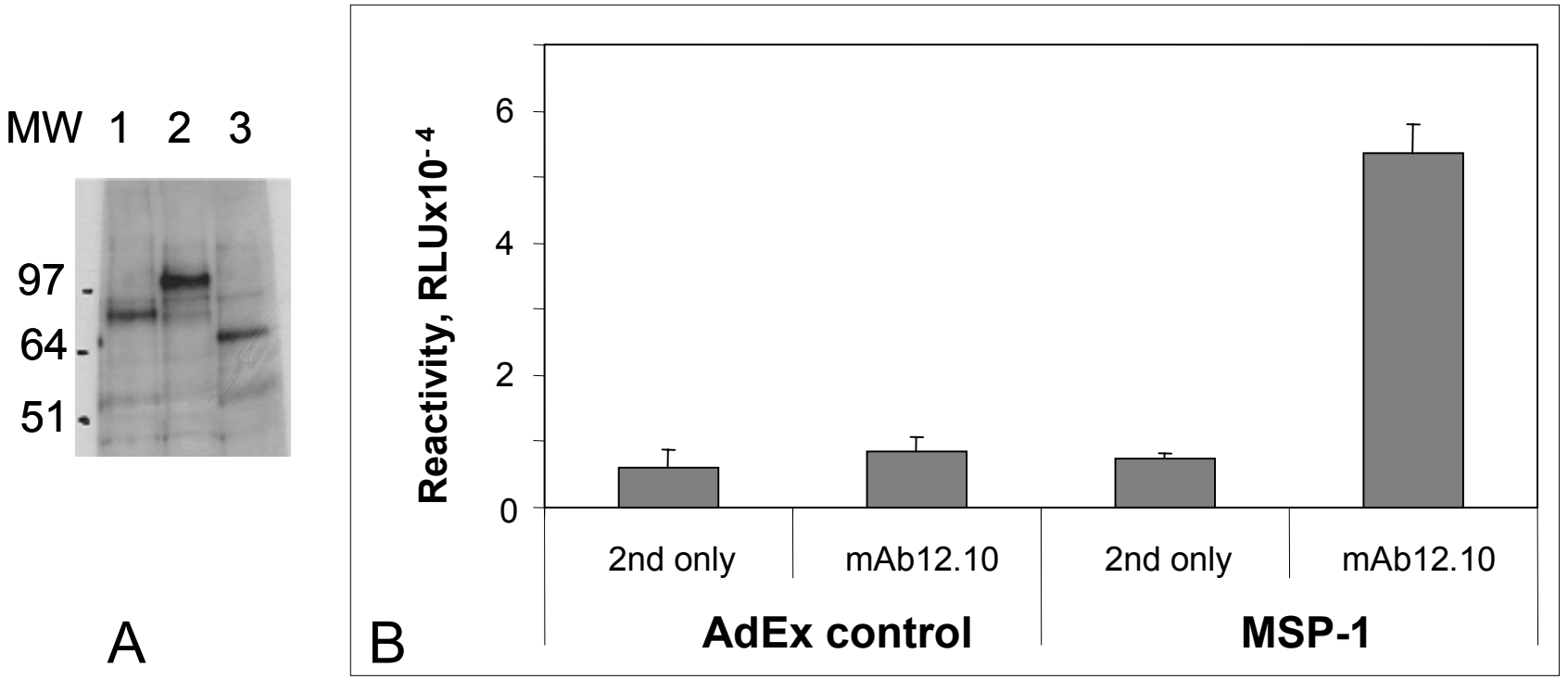

MW 1 2 3
97
64
51
A
Reactivity, RLUx10-4
6
4
2
0
2nd only
mAb12.10
2nd only
mAb12.10
AdEx control
MSP-1
B

Figure 3

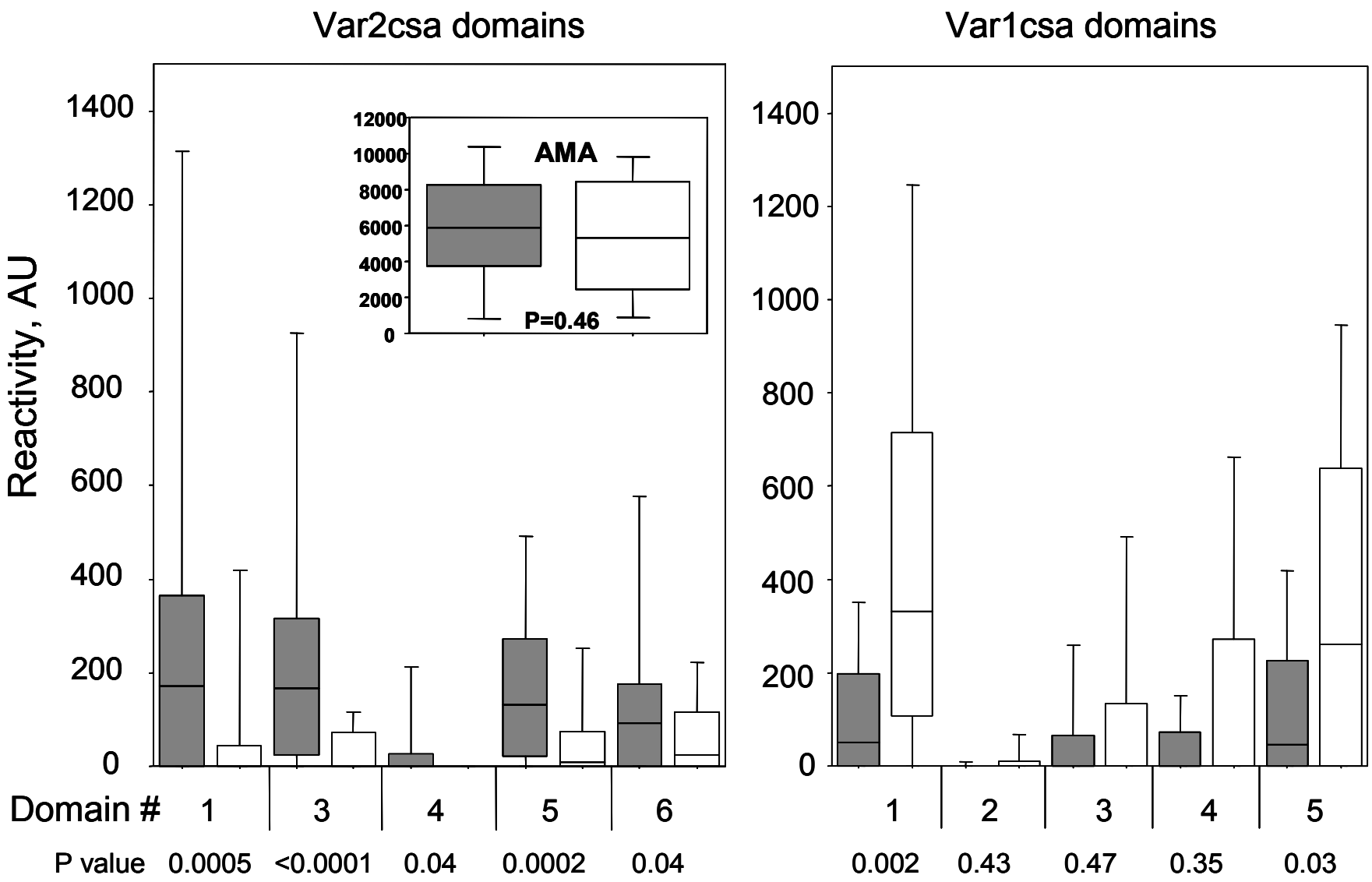
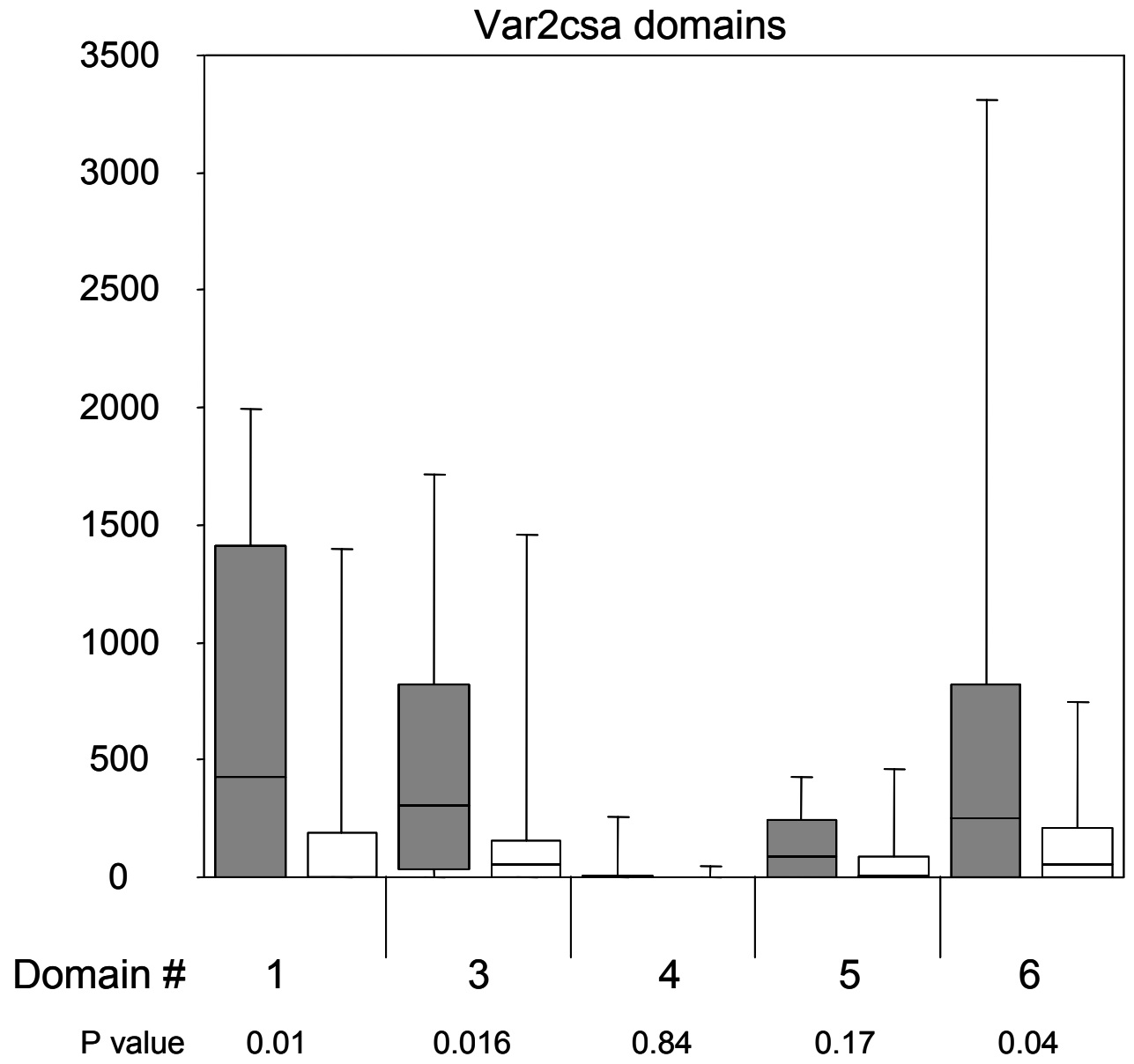

Var2csa domains
Var1csa domains
Reactivity, AU
1400
1200
1000
800
600
400
200
0
12000
10000
8000
6000
4000
2000
0
AMA
P=0.46
Domain #
1
3
4
5
6
P value
0.0005
<0.0001
0.04
0.0002
0.04
1
2
3
4
5
0.002
0.43
0.47
0.35
0.03

Figure 4


Var2csa domains
3500
3000
2500
2000
1500
1000
500
0
Domain #
1
3
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5
6
P value
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0.016
0.84
0.17
0.04

Figure 5

DBL6ε domains

Figure 6A

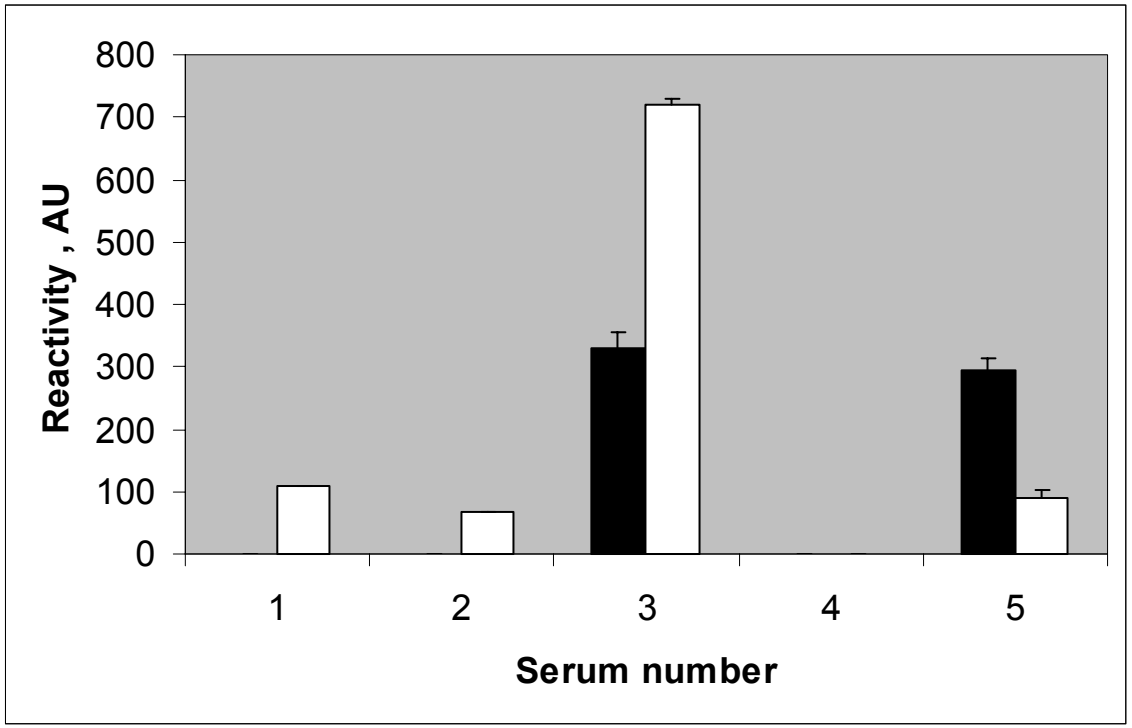
800
700
600
500
400
300
200
100
0
Reactivity , AU
1
2
3
4
5
Serum number

Figure 6B